

## Patients with systemic vasculitis have increased levels of autoantibodies against oxidized LDL

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### SUMMARY

Oxidation of low density lipoprotein (LDL) is considered to play an important role in the development of atherosclerosis and increased levels of autoantibodies against oxidized LDL have been found in patients with various manifestations of atherosclerosis. Patients with vasculitis are prone to the development of atherosclerosis. Since production of radical oxygen species in these patients may result in increased production of oxidized LDL (Ox-LDL), we hypothesized that antibodies against Ox-LDL are elevated during lesion development in vasculitis. Therefore we measured anti Ox-LDL antibodies in 25 patients with ANCA-associated vasculitis and in 42 healthy controls using an enzyme-linked immunosorbent assay (ELISA) in which malondialdehyde modified LDL (MDA-LDL) was coated on microtitre plates. Anti Ox-LDL antibodies were significantly higher in patients as compared to controls ( $P = 0.0001$ ). Anti Ox-LDL levels were also measured in 11 patients during active disease and in these same patients during complete remission. Anti Ox-LDL levels were significantly higher in patients during active disease than during full remission ( $P = 0.001$ ). Our results suggest that patients with ANCA-associated vasculitis are more susceptible to oxidation of LDL, which may contribute to accelerated atherosclerosis development.

**Keywords** oxidized LDL vasculitis autoantibodies atherosclerosis

### INTRODUCTION

Systemic vasculitis is a clinicopathologic process characterized by inflammation and necrosis of bloodvessels [1]. Patients with systemic vasculitis have a higher risk to develop atherosclerosis than healthy controls [2,3]. This finding is supported by animal models in which accelerated atherosclerosis occurred in animals with vasculitis [4]. Atherosclerosis is a complex chronic and progressive disease of the arterial vasculature that consists of the formation of fibrofatty and fibrous lesions, preceded and accompanied by inflammation [5]. Oxidized low density lipoproteins (Ox-LDLs) are believed to play an important role in the progression of atherosclerosis [6]. Ox-LDL is rapidly taken up by macrophages leading to foam cell formation. Furthermore, Ox-LDL is chemotactic for circulating monocytes and T-lymphocytes and promotes cytotoxicity to endothelial cells [7,8].

Production of radical oxygen species plays a pivotal role in the pathophysiology of antineutrophil cytoplasmic antibodies (ANCA) associated vasculitides [9]. These reactive oxygen radicals could be responsible for increased oxidation of LDL

which may lead to accelerated atherosclerosis in these patients. Oxidative modification of LDL induces immunogenic epitopes in the LDL molecule, which results in the formation of autoantibodies against oxidized LDL [10,11]. Increased antibody levels against Ox-LDL have been demonstrated in patients with various manifestations of atherosclerosis and were found to be predictive of the progression of carotid atherosclerosis [12–15]. We hypothesize that patients with ANCA associated vasculitis undergo increased oxidation of LDL and hence have elevated levels of autoantibodies against Ox-LDL during the active phase of their disease. Therefore the aim of the study was to relate the autoantibody levels against oxidized LDL to disease activity in patients with ANCA-associated vasculitis.

### METHODS

#### *Subjects*

Twenty-five consecutive patients (14 men and 11 women;  $53.3 \pm 18.0$ -year-old) with ANCA-associated vasculitis were included in the study. Twenty-one were diagnosed as having Wegener granulomatosis (WG) and 4 were diagnosed as having microscopic polyangiitis (MPA), based on clinical and histopathological criteria [16]. All patients were positive for ANCA. In 18 patients, ANCA were directed to proteinase 3 (Pr3) and in 8

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patients to myeloperoxidase (MPO). Eleven of these patients were also studied after treatment with immunosuppressive agents when complete remission was achieved. Forty-two age-matched healthy subjects (20 men and 22 women;  $51.3 \pm 15.1$  years old) served as the control group. None of the controls had a history of vasculitis or atherosclerosis.

#### LDL isolation and MDA-LDL-preparation

LDL was isolated from plasma of a healthy subject by ultracentrifugation in a KBr discontinuous gradient according to Redgrave *et al.* [17]. KBr and EDTA were removed by rapid filtration through disposable desalting columns (Econo-Pac 10 DG, Bio-Rad). The LDL protein content was determined according to Lowry *et al.* [18]. Native LDL was either freshly used for preparation of MDA-LDL or stored at 4°C under N<sub>2</sub> with 1 mg/ml EDTA as preservative.

MDA-LDL was prepared as described by Palinski *et al.* [19] and was stored at 4°C under N<sub>2</sub>.

#### Enzyme-linked immunosorbent assay (ELISA) for antibodies against oxidized LDL

For the measurement of antibodies against Ox-LDL by ELISA, 48 wells of a disposable 96 well polystyrene plate (Nalge Nunc International, Roskilde, Denmark) were coated with native LDL and the other 48 were coated with MDA-LDL, both at concentrations of 100 µg/ml in PBS. The plates were kept at 4°C overnight and then washed five times with a washing buffer containing 0.01 M Tris-HCl (pH 8.0), 0.15 M NaCl and 0.05% Tween 20 in H<sub>2</sub>O. After washing, serum samples diluted in incubation buffer containing 0.1 M Tris HCl, 0.3 M NaCl and 0.05% Tween 20 in H<sub>2</sub>O (1 : 100) were incubated in triplicate at 4°C overnight. Furthermore, a dilution curve of a positive serum sample (1 : 12.5; 1 : 25; 1 : 50; 1 : 100; 1 : 200; 1 : 400; 1 : 800; 1 : 1600) was used for calibration and standardization.

After washing five times with washing buffer, alkaline phosphatase conjugated goat antihuman IgG F (ab)<sub>2</sub> (American Qualex, San Clemente, CA, USA) diluted 1 : 3000 in incubation buffer was added to each well and incubated for 1 h at 38°C. After washing five times with washing buffer, 100 µl of freshly made substrate (phosphatase substrate tablets; Sigma, St. Louis USA) was added in each well and incubated for 30 min at 38°C. The reaction was stopped with 100 µl of 5N NaOH and colour was read at 405 nm. Results were expressed as anti Ox-LDL levels from triplicate determinations and were calculated by subtracting binding to native LDL from binding to MDA-LDL. The OD value of the 1 : 100 dilution of the reference sample was set at 100 arbitrary units (1 : 200 = 50 arbitrary units, etc.). The intra-assay coefficient of variation between triplicate tests was 3.3% and the interassay coefficient was 10.6%. Results were also expressed as binding to MDA-LDL and LDL (absorption units) and as the ratio MDA-LDL/LDL. All results were obtained with native and MDA modified LDL preparations that were stored less than two weeks.

#### Statistical analysis

Anti Ox-LDL levels of patients with vasculitis and of the controls were compared using the Mann–Whitney *U*-test. The Wilcoxon signed rank test was used to compare anti Ox-LDL levels of patients with active disease and during complete remission. Bonferroni correction was applied to correct for type I errors.  $P < 0.05$  was considered statistically significant.

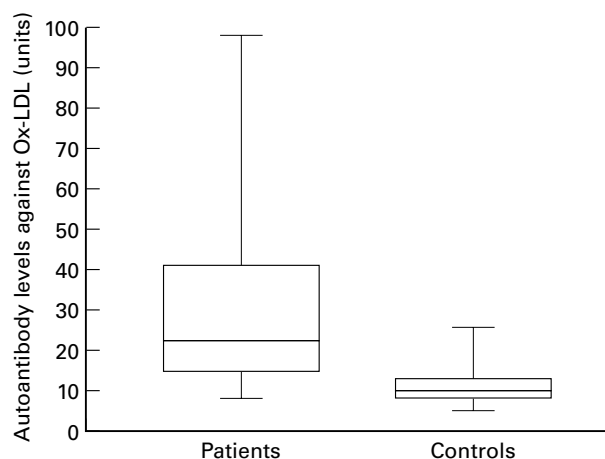
## RESULTS

Figure 1 shows a box plot of the anti Ox-LDL antibody levels of patients with active ANCA-associated vasculitis and the healthy controls. Anti Ox-LDL antibodies were significantly higher in patients with active ANCA-associated vasculitis as compared to the levels in healthy controls ( $29.62 \pm 21.31$  versus  $10.93 \pm 4.24$  arbitrary units, respectively (mean  $\pm$  SD);  $P < 0.0001$ ). Autoantibody levels against Ox-LDL were significantly higher in patients during active disease compared to the levels in the same patients during complete remission ( $30.82 \pm 24.66$  versus  $10.68 \pm 3.28$  arbitrary units, respectively;  $P = 0.001$ ). No significant difference was found between anti Ox-LDL levels in patients with ANCA-associated vasculitis during remission and the levels in controls ( $P = 0.9388$ ). Figure 2 shows the changes in levels of autoantibodies against Ox-LDL in these patients during active disease and at the time of remission.

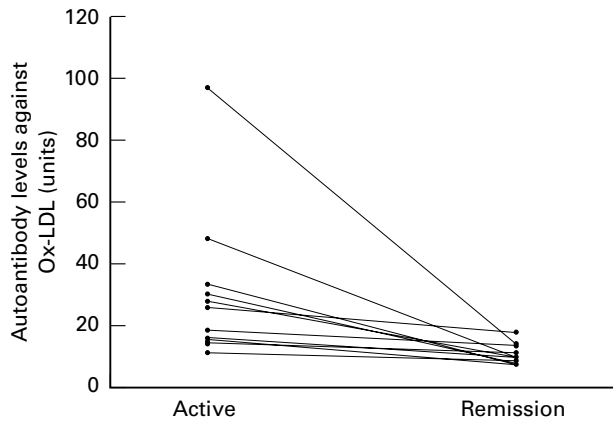
Figure 3 shows the binding to MDA-LDL, native LDL and the ratio MDA-LDL/native LDL (absorption units) in serum from controls, patients with active disease and patients after remission. Binding to MDA-LDL and to LDL and the ratio MDA-LDL/LDL were significantly higher ( $P < 0.0001$ ,  $P < 0.0001$ ,  $P = 0.016$ , respectively) in patients with active disease ( $0.52 \pm 0.18$ ,  $0.18 \pm 0.07$  and  $2.83 \pm 0.66$ , respectively) as compared to apparently healthy controls ( $0.30 \pm 0.06$ ,  $0.13 \pm 0.03$ , and  $2.37 \pm 0.40$ , respectively). Also, these values were significantly higher in 11 patients during active disease ( $0.49 \pm 0.15$ ,  $0.16 \pm 0.03$  and  $2.94 \pm 0.57$  for MDA-LDL, LDL and MDA-LDL/LDL, respectively) compared to the levels in the same patients during complete remission ( $0.30 \pm 0.08$ ,  $0.13 \pm 0.01$  and  $2.37 \pm 0.50$ , respectively;  $P < 0.01$ ). No significant differences were found between binding to MDA-LDL and to LDL and the ratio MDA-LDL/LDL in patients with ANCA-associated vasculitis during remission and controls.

## DISCUSSION

In our study, we found high levels of anti Ox-LDL antibodies in



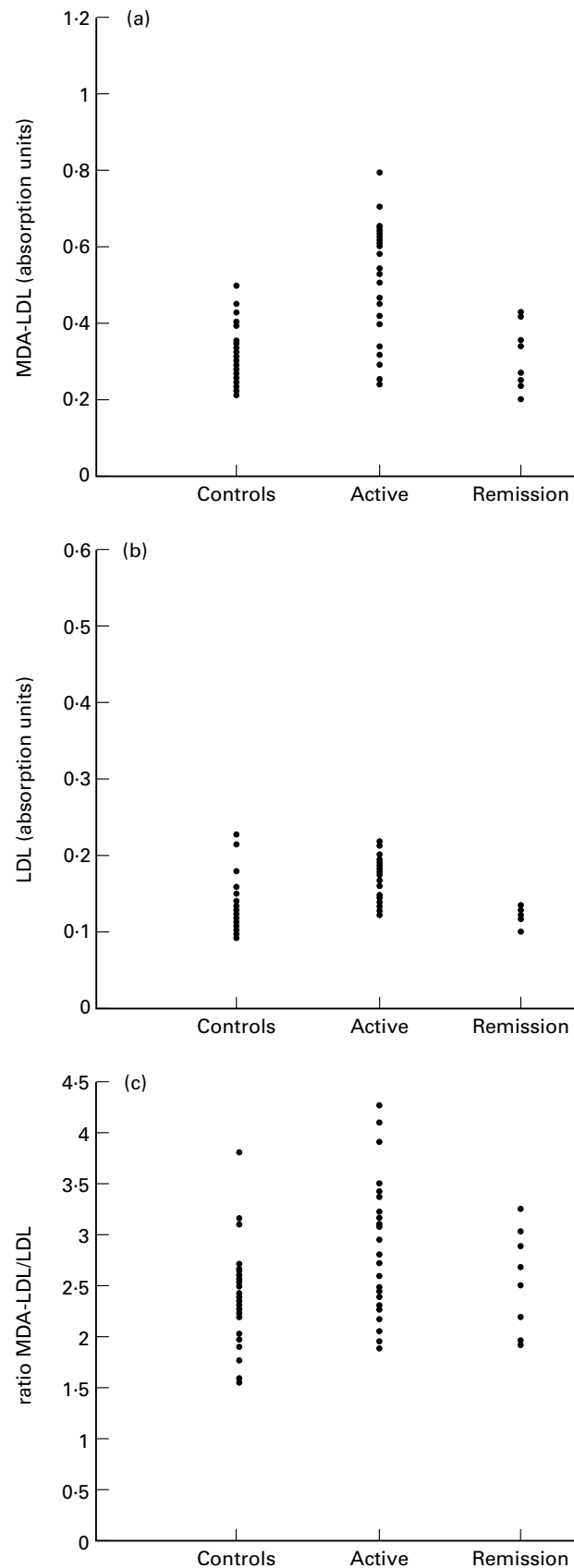
**Fig. 1.** Box plot of autoantibody levels against Ox-LDL in patients with ANCA-associated vasculitis during active disease ( $n = 25$ ) and healthy controls ( $n = 42$ ). The box includes observations from the 25th to the 75th percentile. The horizontal line within the box represents the median value. Lines outside the box represent the highest and lowest value. Autoantibody levels against Ox-LDL were significantly higher in patients compared to levels in controls ( $P < 0.0001$ ).



**Fig. 2.** Changes in levels of autoantibodies against Ox-LDL in 11 patients with ANCA-associated vasculitis. During active disease, antibody levels were significantly higher than at the time of remission ( $P = 0.001$ ). Each line represents data of an individual patient.

patients with an active form of ANCA-associated vasculitis as compared to controls. Anti Ox-LDL levels were significantly higher during active disease as compared to complete remission. Elevated serum levels of anti Ox-LDL antibodies have been reported in other autoimmune diseases that are associated with accelerated atherosclerosis such as systemic lupus erythematosus [20,21]. To our knowledge, however, the occurrence of autoantibodies to epitopes of oxidized LDL in patients with ANCA-associated vasculitis has not been described previously. The presence of these autoantibodies may be due to the increased production of reactive oxygen radicals, which plays a pivotal role in the pathophysiology of ANCA-associated vasculitis. Recently we proposed a hypothetical pathophysiological scenario for this disease [9]. In short, microbial agents induce the release of cytokines such as interleukine 1 (IL-1) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). These cytokines induce the expression of the target antigens of ANCA, i.e. Pr3 and MPO on the cell membrane of granulocytes and monocytes [9,22]. Subsequently, ANCA bind to their target antigens, which induces activation of granulocytes and monocytes resulting in the production of reactive oxygen radicals and release of lysosomal enzymes, leading to endothelial cell damage [9,22–24].

LDL entering the subendothelial space could be oxidized by these reactive oxygen radicals released by the ANCA stimulated granulocytes and monocytes. In addition, activated macrophages, endothelial cells and smooth muscle cells may contribute to the oxidation of LDL [25]. Subsequently, the increased oxidation of LDL may result in increased production of adhesion molecules such as E-selectin and vascular cell adhesion molecule (VCAM) and increased secretion of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage colony-stimulating factor (MCSF) by endothelial cells [26,27]. Altogether, these factors may result in increased adherence and migration of leucocytes, which may promote both the vasculitic and the atherosclerotic process in these patients.



**Fig. 3.** Binding to (a) MDA-LDL, (b) native LDL and (c) the ratio MDA-LDL/LDL in healthy controls (Controls,  $n = 42$ ), patients with ANCA-associated vasculitis during active disease (Active,  $n = 25$ ) and patients with ANCA-associated vasculitis after remission (Remission,  $n = 11$ ).

Patients during active disease have higher anti Ox-LDL levels than during complete remission. Also, significantly lower ANCA titres were found in patients during remission (data not shown). Previously, Mulder *et al.* [28] found that neutrophils produce less oxygen radicals if stimulated *in vitro* by ANCA containing IgG fractions from patients during remission as compared to stimulation by ANCA containing IgG fractions derived from patients during an active phase of disease. Therefore, we hypothesize that during remission less oxygen radicals are produced, less LDL is oxidized and, hence, less antibodies against Ox-LDL are found.

Whether antibodies to Ox-LDL play a modulating role in the atherosclerotic process is at present still controversial [29]. Titres of antibodies to Ox-LDL have been found to be highly predictive of the rate of progression of atherosclerosis and have been found to be an independent risk factor predictive of progression of the disease [15,29]. Furthermore, a significant correlation between titres of anti Ox-LDL antibodies and the extent of atherosclerosis has been found in LDL receptor deficient mice [30]. From these data we hypothesize that accelerated atherosclerosis in patients with vasculitis might be due to persistent activity of the disease resulting in persistent production of oxidized LDL and antibodies to Ox-LDL. Decrease of autoantibodies to Ox-LDL to normal levels during remission suggests that this accelerated atherosclerosis may be circumvented by aggressive treatment of the vasculitic disease process, which results in a complete remission of the disease and hence decreased oxidation of LDL and decreased production of antibodies to oxidized LDL.

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